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# Production of maltodextrin 1-Phosphate by *Fibrobacter succinogenes* S85

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**Abstract** We show for the first time the occurrence of maltodextrin-1-Phosphate (MD-1P) (DP  $\geq 2$ ) in *F. succinogenes* S85, a rumen bacterium specialized in cellulolysis which is not able to use maltose and starch. MD-1P were found in intra and extracellular medium of resting cells incubated with glucose. We used 2D  $^1\text{H}$  NMR technique and TLC to identify their structure and quantify their production with time. It was also shown that these phosphorylated oligosaccharides originated both from exogenous glucose and endogenous glycogen.

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**Keywords:** Rumen; Maltodextrin; Maltodextrin-phosphate; NMR; *Fibrobacter succinogenes*

## 1. Introduction

*Fibrobacter succinogenes* S85, a cellulolytic rumen bacterium, degrades cellulose into cellobiose and glucose that are metabolized and fermented to succinate and acetate [1–4]. The metabolism of glycogen in this strain appeared to play an important role and we showed that this polysaccharide was subjected to a cycle [1,3]. We also found that the bacteria metabolizing glucose were able to synthesize and release oligosaccharides identified by 2D NMR techniques as maltodextrins (MD) [5]. This fact was surprising because strain S85 of *F. succinogenes* was not known to be able to use starch or maltose [6]. The MD were found in the millimolar concentration range in the extracellular medium and they had a maximal degree of polymerization of about 6–7 units.

In the present work, we show for the first time the occurrence of maltodextrin-1-Phosphate (degree of polymerization, DP  $\geq 2$ ) in cells and extracellular medium of *F. succinogenes* S85 resting cells incubated with glucose. We used 2D NMR techniques and TLC to identify maltodextrin-1-Phosphate structure, to study the kinetics of their production and their

origin. Note that the use of NMR techniques, which represents a new approach in this field, allowed simultaneous qualitative and quantitative analysis of these oligosaccharides.

## 2. Materials and methods

### 2.1. Preparation of cells, extracellular and cellular media

*Fibrobacter succinogenes* S85 (ATCC 19169) was grown for 15 h in a chemically defined medium [2] with 3 g l<sup>-1</sup> cellobiose. Cells harvested in the late log phase were centrifuged (6000  $\times$  g, 10 min, 4 °C) and re-suspended under a 100% CO<sub>2</sub> atmosphere in a reduced 50 mM potassium phosphate buffer containing 0.4% Na<sub>2</sub>CO<sub>3</sub>, 0.05% cysteine and 13 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at pH 7.1 [4]. Cell suspensions (5 mg protein ml<sup>-1</sup>) were incubated with 32 mM glucose at 38 °C in a water bath. At regular intervals (10 min), 3.5 ml of the cell suspension was taken, centrifuged (13 000  $\times$  g, 10 min, 4 °C), and the supernatant kept as extracellular medium. The pellet was resuspended in water and cells were broken by successive freeze thawing. Cell debris was eliminated by centrifugation (14 000  $\times$  g, 15 min) and the supernatant was assimilated to cellular medium (cytoplasm and periplasm). We checked that no cell lysis occurred during the incubations by measuring the activity of L-glutamate dehydrogenase (as described in [7]), a cytoplasmic marker, in the extracellular medium and by comparing it with that of corresponding cell extracts.

### 2.2. NMR spectroscopy

Measurements of NMR spectra were performed at 27 °C on a 300 or 500 MHz Avance Bruker spectrometer equipped with 5mm TXI  $^1\text{H}$ ,  $^{13}\text{C}$ ,  $^{15}\text{N}$  probe with inverse detection. pH of cell-free supernatants or intracellular media was corrected to 7.40. Samples were freeze-dried twice with D<sub>2</sub>O and further dissolved in a mixture of 470  $\mu\text{l}$  of 99.98% D<sub>2</sub>O and 20  $\mu\text{l}$  of 10 mM sodium 3-(trimethylsilyl)propionate-d<sub>4</sub> (TSP-d<sub>4</sub>), which was used as internal reference for chemical shift and quantitation.

Basic quantitation of H1 signals at  $\delta$  5.46 and 5.43–5.41 (each representing a mixture of metabolites, species) was performed in the  $^1\text{H}$  NMR spectra. Obtained values served as the reference for calculation of metabolite concentrations based on H1/H2 cross-peaks integrals taken in gradient enhanced double quantum filtered  $^1\text{H}$ – $^1\text{H}$  correlated COSY (ge-DQF COSY) spectra (standard Bruker program). Because of the identical H1/H2 chemical shifts of free glucose-1-Phosphate (Glc-1P) and MD-1P<sub>a</sub> (Glc-1P part of maltodextrin-1-Phosphate (MD-1P) molecule) for the calculation of free Glc-1P concentration, a very clearly separated cross-peak signal of MD-1P<sub>b</sub> ( $\delta$  5.43/3.58, due to the Glc unit next to Glc-1P in MD-1P) was used.

### 2.3. Thin layer chromatography

TLC was carried out as described in [4] using a mixture of glucose, maltose, maltotriose and maltotetraose, or phosphorylated sugars (3 g l<sup>-1</sup>) as a standard. Spot 1 and Spot 2 were scraped from the plate and eluted with water. After centrifugation to eliminate the silica, the supernatant was freeze-dried and dissolved in 99.98% D<sub>2</sub>O twice and sealed in NMR tube for NMR analysis.

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**Abbreviations:** Glc, glucose; Glc-1P, glucose-1-Phosphate; Glc-6P, glucose-6-Phosphate; MD-1P, maltodextrin-1-Phosphate; MD-1P<sub>a</sub>, Glc-1P unit of MD-1P; MD-1P<sub>b</sub>, Glc unit neighboring Glc-1P of MD-1P; MD, linear maltodextrins; MD<sub>t</sub>, terminal Glc unit of MD; MD<sub>int</sub>, internal Glc units of MD and MD-1P;  $\alpha\text{MD}_r$  and  $\beta\text{MD}_r$ , maltodextrin reducing end  $\alpha\text{Glc}$  and  $\beta\text{Glc}$  unit, respectively; ge-DQF COSY, gradient enhanced double quantum filtered  $^1\text{H}$ – $^1\text{H}$  correlated COSY; DP, degree of polymerization

#### 2.4. Enzyme and BaCl<sub>2</sub> treatment

Amyloglucosidase treatment: the sample was incubated with 80 µg/ml amyloglucosidase (1,4,α-D-glucan glucohydrolase; EC 3.2.1.3; from *Rhizopus* genus mould) for 60 min at 55 °C in 50 mM potassium phosphate buffer (pH 4.5). β-amylase and maltase treatment, Sample pH was adjusted to 4.5. 10 µl of β-amylase (1, 4,α-D-glucan maltohydrolase; EC 3.2.1.2; this enzyme hydrolyzes (1,4)α-glycosidic linkages and removes successive maltose units from the non-reducing ends of the chains) was added to the sample that was then incubated for 30 min at 20° C. 1 ml of this mixture was analyzed by <sup>1</sup>H NMR and the other 1 ml was subsequently treated with 20 µl maltase (1,4,α-D-glucan glucohydrolase, EC 3.2.1.20; this enzyme removes terminal glucose units α-linked to oligosaccharides) after adjusting the pH to 6.8, for 30 min at 38 °C.

BaCl<sub>2</sub> precipitation of phosphorylated sugars: concentrated extracellular medium or cellular extracts were added with BaCl<sub>2</sub> at 0.8 and 0.4 mM, respectively, and were kept at 4 °C for 15 h. The soluble fraction was then lyophilized and analyzed by NMR. The precipitate was resolubilized by adding water and Dowex 50X8 resin to replace Ba<sup>+</sup> by Na<sup>+</sup> ions. After 24 h agitation, the solubilized products were freeze-dried and analyzed by NMR.

#### 2.5. Metabolite assays

Protein concentration was determined by the Bradford method. Succinate, acetate, and glucose were assayed using enzymatic kits (Roche). MD, MD-1P, and Glc-1P were quantified from <sup>1</sup>H NMR spectra.

#### 2.6. Chemicals

TSP-d<sub>4</sub> was purchased from Eurisotop (France). Glucose, maltose, maltotriose, maltotetraose, Glc-1P and glucose-6-Phosphate (Glc-6P) were from Sigma. All enzymes and chemicals were from Sigma or Roche.

### 3. Results

Resting cells of *F. succinogenes* were incubated with 32 mM glucose. Samples of the cell suspension were taken at regular time intervals and extracellular and intracellular media were separated by centrifugation. 18–20 mM succinate and 7–8 mM acetate were produced in the extracellular medium after 40 min, while 26–27 mM glucose were consumed. The extracellular and intracellular media were analyzed in parallel by 2D <sup>1</sup>H NMR spectroscopy and by TLC and showed the unexpected occurrence of MD-1P.

#### 3.1. Structural identification of MD-1P

<sup>1</sup>H chemical shifts of identified metabolites are reported in Table 1. Figs. 1A and C present regions of ge-DQF COSY NMR spectra of H1/H2 cross-peaks for sugars of interest in the extracellular medium and cell extracts, respectively. In addition to previously identified maltodextrins and glucose-1-P, a cross-peak at δ 5.43/3.58 was assigned to a terminal non-reducing end Glc unit of maltose-1P, as the main component, and to Glc residues neighboring the Glc-1P unit of maltodextrin-1P of longer chains (Fig. 1, Glc unit neighboring Glc-1P of MD-1P (MD-1P<sub>b</sub>)). Note that in the rest of the text, MD-1P will stand for both maltose-1P and maltodextrin-1P. The H1/H2 chemical shifts (δ 5.46/3.52) of the Glc-1P part of the maltose-1P molecule (Fig. 1, MD-1P<sub>a</sub>) are identical to the H1/H2 chemical shifts of free Glc-1P. The assignment of MD-1P<sub>a</sub> and MD-1P<sub>b</sub> signals to maltodextrin-1P is supported by the following facts: (i) chemical shifts of these resonances are in agreement with those of maltose-1P (Fig. 1B); (ii) ge-DQF COSY spectra of samples obtained with various incubation times showed that intensities of both MD-1P cross-peaks (δ 5.43/3.58 and δ 5.46/3.52) changed simultaneously with the

Table 1

Chemical shifts of sugar metabolites identified in supernatants and cell extracts in D<sub>2</sub>O at 27 °C, pH 7.4

Metabolites	Chemical shifts (δ ppm)	
	H1	H2
αGlc	5.24	3.54
βGlc	4.65	3.24
αMD <sub>r</sub>	5.24	3.58
βMD <sub>r</sub>	4.66	3.28
MD <sub>int</sub> <sup>a</sup>	5.41	3.63
MD <sub>i</sub>	5.41	3.59
αGlc-6P	5.24	3.58
βGlc-6P	4.65	3.28
Glc-1P	5.46	3.52
MD-1P <sub>a</sub>	5.46	3.52
MD-1P <sub>b</sub>	5.43	3.58

<sup>a</sup>These chemical shifts have been re-assigned compared to those previously published [5]: H1 δ 5.44; H2: δ 3.57.

intensities of neighboring terminal Glc unit of MD (MD<sub>i</sub>) + internal Glc units of MD and MD-1P (MD<sub>int</sub>) cross-peaks: this fact indicated that they should be related to each other; iii) when the supernatant samples were added with BaCl<sub>2</sub>, which is known to precipitate phosphorylated species, both signals assigned to MD-1P disappeared from the spectra and only signals of non-substituted MD could be detected (Fig. 1D). In addition, in the <sup>1</sup>H NMR spectrum as well as ge-DQF COSY spectrum of precipitated phosphorylated species, not only both signals due to maltose-1P at δ 5.46/3.52 and 5.43/3.58 but also the low intensity signal at δ 5.41 characteristic of internal glucose units of MD were detected, indicating the occurrence of small amounts of phosphorylated MD of DP > 2 (not shown).

Fig. 2 shows the results of thin layer chromatography experiments (TLCs) of supernatants and cell extracts obtained with incubations of *F. succinogenes* resting cells with glucose (Fig. 2A). In parallel to the occurrence of maltodextrins of DP from 3 to 7, in agreement with NMR data [5] and the nature of which was confirmed by treatment with amyloglucosidase (EC 3.2.1.3.) (Fig. 2C a and b), the occurrence of two large spots (Spot 1 and Spot 2) was detected. Spot 1 was present in both supernatants and cell extracts, while Spot 2 was found only in the supernatant. Their R<sub>f</sub> were consistent with that of phosphorylated sugars (Glc-1P and Glc-6P, Fig. 2D). When the samples were added with BaCl<sub>2</sub> to precipitate phosphorylated species Spot 1 and Spot 2 disappeared on TLC (as shown for Spot 1 in Fig. 2C c and d). In parallel, Spot 1 was eluted with water from TLC obtained from cell extracts or supernatants, and analyzed by 2D and 1D NMR experiments. Glc-6P was the main component of the mixture, while MD-1P and free Glc-1P were present in smaller amounts in both the supernatants and the cell extracts. Other unidentified compounds were also present. In addition, eluted Spot 1 was treated by phosphorylase and analyzed again by TLC. Spots of maltodextrins up to DP 6 appeared, confirming the occurrence of maltodextrins-1P in the mixture (not shown).

Finally, the occurrence of MD-1P was confirmed by another complementary experiment. The supernatant obtained from the incubation of *F. succinogenes* resting cells with glucose was submitted to various enzymatic treatments. The samples were then analyzed by <sup>1</sup>H NMR spectra (Fig. 3): spectrum A corresponds to the non-treated sample. Spectrum B shows the

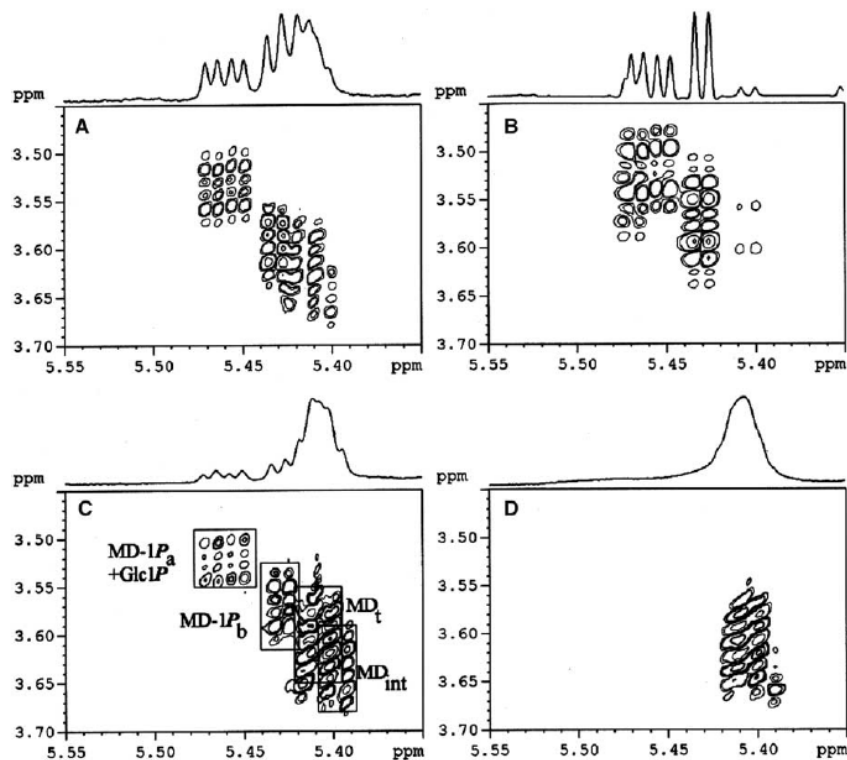


Fig. 1.  $\alpha$ -Anomeric region of ge-DQF COSY spectra showing H1/H2 cross-peaks. Supernatant (A) and cell extract (C) obtained after 30-min incubation of *F. succinogenes* resting cells; (B) maltose-1P; (D) supernatant after precipitation by  $\text{BaCl}_2$ .

effect of the treatment with  $\delta$ -amylase (EC 3.2.1.2.), an exo-enzyme cleaving Glc unit from the non-reducing end of higher MD, finally yielding maltose. The broad signal of

$\text{MD}_t + \text{MD}_{\text{int}}$  ( $\delta$  5.41) disappeared and a new shoulder appeared due to the non-reducing Glc unit of maltose at  $\delta$  5.41, while both anomeric signals due to maltose-1P ( $\delta$  5.46 and

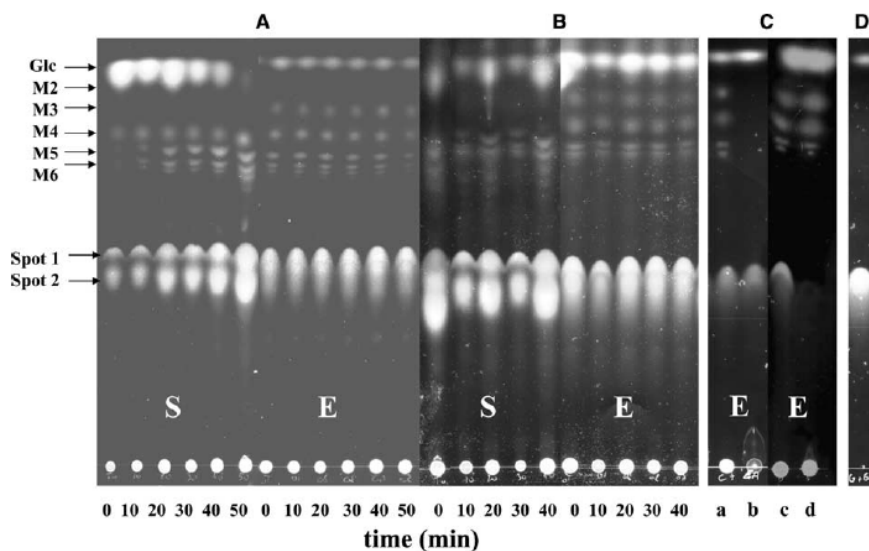


Fig. 2. TLC of saccharide derivatives present in supernatants (S) or cell extracts (E). Incubations of *F. succinogenes* resting cells (A) with 32 mM glucose; (B) without any exogenous carbon substrate. (C) Cellular extract samples taken at 40 min before (a) and after (b) treatment by amyloglucosidase; similar samples before (c) and after (d) treatment by  $\text{BaCl}_2$ . (D) Standards: mixture of Glc-1P and Glc-6P. Glc, glucose; M2, maltose; M3, maltotriose; M4, maltotetraose; M5, maltopentaose; M6, maltohexaose; Spot 1, Spot 2, spots containing phosphorylated sugar species. Remark: the same amount was sampled on TLCs, however, the image presented in B was treated with a higher intensity and contrast in order to enhance the intensity of various spots.

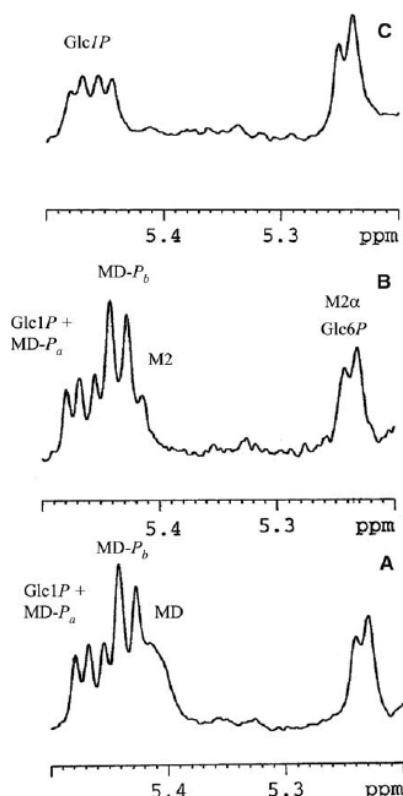


Fig. 3.  $^1\text{H}$  NMR spectra (300 MHz) of supernatants. Incubation with 32 mM Glc (A), after its treatment with  $\beta$ -amylase (B), after its treatment with  $\beta$ -amylase and maltase (C).

5.43) remained untouched. After the subsequent treatment with maltase (EC 3.2.1.20), which cleaves terminal glucose in  $\delta$ -1,4-linked oligosaccharides, only the Glc1P signal could be observed (spectrum C).

### 3.2. Quantitation of MD-1P and kinetics of their production

The resolution in the  $^1\text{H}$  NMR spectra allowed us to carry out an integration of MD-1P signals in the region  $\delta$  5.50–5.38 ppm and thus to compare their concentration to that of Glc-1P and MD (estimated MD value includes the contribution of internal Glc units of longer maltodextrin-1P present in low quantities). The time course of sugar concentration changes is presented in Fig. 4A and B. MD-1P quantitation was made by integration of MD-1P<sub>b</sub>  $^1\text{H}$  NMR signal. The MD-1P concentration in supernatants was within the range 0.2–0.3 mM, while in cell extracts the measured concentration in NMR tubes was around 0.15 mM, which corresponds to a calculated intracellular concentration of about 4.5 mM considering an intracellular volume of 5.5  $\mu\text{l}/\text{mg}$  of protein [8]. MD-1P intracellular and extracellular concentrations were slightly higher than those of Glc-1P (up to two fold in the extracellular medium). In comparison, MD concentrations were much higher: 0.4–1.3 mM in the extracellular medium (measured), 20–36 mM in the intracellular medium (calculated). The MD concentration in supernatants (Fig. 4A) increased gradually until the end of incubation, while in the cell extracts concentrations were relatively high at the beginning of incubation, reached a maximum after 20 min and then gradually decreased (Fig. 4B).

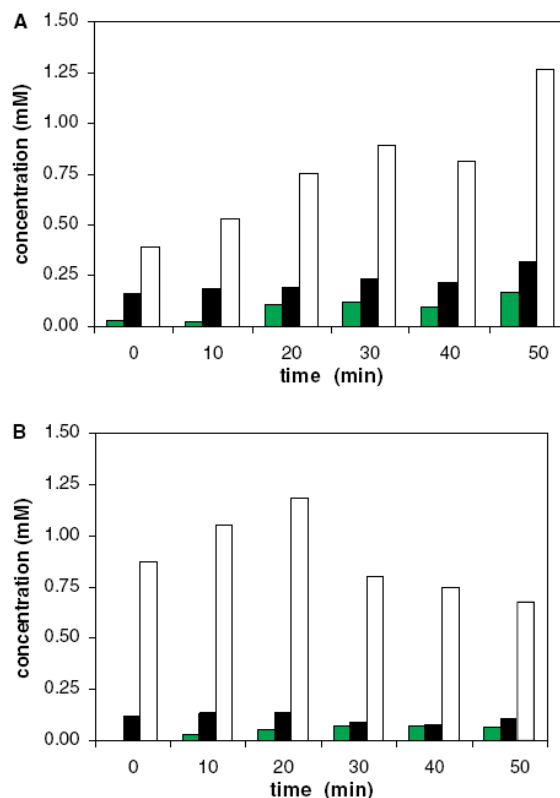


Fig. 4. Time courses of concentrations of MD (empty squares), MD-1P (green squares), Glc-1P (black squares). Supernatants (A) and cell extracts (B) obtained after incubation of *F. succinogenes* resting cells with 32 mM glucose. Concentrations are given as direct values measured in the NMR tubes.

As the intracellular concentration of MD results from both their synthesis and their excretion from the cells, this observation may indicate that the rate of MD synthesis slowed after 20 min. The time course of MD-1P concentration appeared similar to that of MD, although the observed variations were lower. Glc-1P concentrations increased with time both in supernatants and in cell extracts.

The intensity of Spot 1 and Spot 2 increased with time as shown on TLCs (Fig. 2A); this result is consistent with the increase of MD-1P and Glc-1P measured by NMR.

### 3.3. Origin of MD-1P

Fig. 2B shows TLCs of intracellular and extracellular media of cells incubated without any exogenous substrate in which the endogenous glycogen was the only carbon substrate. Comparison of Fig. 2A and B suggests that the same spots are present independently of the carbon source, although the intensity of the spots was much lower in the absence of external glucose (see legend for Fig. 2). This suggests that glycogen may be the source also for the synthesis of MD and phosphorylated compounds identified in Spot 1 (MD-1P, Glc-6P and Glc-1P). This is in agreement with the data obtained by isotopomer analysis ( $^{13}\text{C}$  versus  $^{12}\text{C}$ ) of MD, Glc-1P and Glc-6P previously described in incubations of *F. succinogenes* in the presence of  $[1-^{13}\text{C}]\text{glucose}$  [5]. In the ge-DQF COSY spectra, H1/H2 cross-peaks ( $\delta$  5.71/3.58; 5.15/3.58) due to  $^{13}\text{C}$  satellites of



MD-1P (corresponding to protons linked to  $^{13}\text{C}$ ) are consistent with MD-1P synthesis from exogenous  $^{13}\text{C}$  labeled glucose [5], while the occurrence of the H1/H2 cross-peak ( $\delta$  5.43/3.58) due to non-labeled MD-1P suggest also their simultaneous synthesis from endogenous glycogen.

#### 4. Discussion

The major finding of this paper is the production of MD-1P, consisting mainly of not only maltose-1P but also phosphorylated maltodextrins of longer DP (up to 6), by *F. succinogenes* S85, a rumen bacterium specialized in cellulolysis which is not able to use maltose and starch. The occurrence of maltose-1P has been described in some bacteria growing on maltose, however, the production of longer maltodextrin-1-Phosphate (MD-1P) is described here for the first time. The evidence of MD-1P structure was given by the use of 2D ge-DQF COSY experiments performed directly in situ on intracellular and extracellular media. These compounds were present both in the intra and extracellular media, but the intracellular concentration was 15–20 times higher. Excretion of phosphorylated sugars is unusual, however, we previously showed intracellular accumulation and excretion of Glc-1P and Glc-6P in *F. succinogenes* [2,4,5]. It was also shown that *Escherichia coli* was able to excrete Glc-6P under specific conditions [9].

Our results show that production of MD and MD-1P in *F. succinogenes* is interconnected. First, the kinetics of their production is similar in the cells and the extracellular medium, and these oligosaccharides have the same length. Second, they are both synthesized from exogenous glucose or endogenous glycogen. Synthesis of maltodextrin and particularly maltotriose from endogenous glycogen was previously shown in yeast [10] as well as in *E. coli* mutants in the maltose system [11,12]. Such maltotriose production could occur in *F. succinogenes* as this oligosaccharide is clearly seen on TLCs of cell extracts (Fig. 2) and appears to be of the minimum length of MD. In addition, glucose units of MD and MD-1P could originate from the cycling of glycogen that operates as previously shown in *F. succinogenes* [1,3]. One possibility would be that MD-1P result from MD phosphorylation, thus being end-products. It was recently shown that an ATP-dependent maltokinase is able to phosphorylate not only maltose but also

longer maltodextrins (DP up to 6) in *Actinoplanes missouriensis* [13]; this type of maltokinase could operate in *F. succinogenes* transforming MD into MD-1P. Another possibility would be that MD-1P are intermediates of MD synthesis; however, to our knowledge, no enzyme able to dephosphorylate MD-1P has been described.

The role of maltose-1P and of MD-1P of longer lengths in *F. succinogenes* remains unknown. Moreover, the role of maltose-1P in *E. coli* and in *A. missouriensis* has not been elucidated. Such phosphorylated oligosaccharides could act as inducers of genes or operons as suggested for the *mal* operon in *E. coli* [14]; they could also be involved as signal molecules for intra or inter species communication in the rumen ecosystem. Further work is needed to identify and characterize the enzyme involved in MD-1P synthesis in *F. succinogenes* and also to understand the role of these molecules.

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